

# Factors that cause the appearance of ghost bands when using chemiluminescent detection systems in a Western blot

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Application Note # 11  
AN0011.2

August 2005

## Abstract

We demonstrate several conditions that cause the appearance of halo or ghost-like protein bands in Western blots. Researchers attempting to adapt to extremely sensitive chemiluminescent detection systems commonly encounter this problem. Using several concentrations of purified proteins, we varied different Western blot factors including secondary antibody concentration, substrate amounts and incubation times, and the percentage of non-fat milk in the blocking solution. Our results indicate that the most common causes for ghost-band effects are overloading the gel with excess target protein and using antibodies that cross-react to component(s) of the blocking solution.

## Introduction

The transition from traditional colorimetric detection to extremely powerful newer chemiluminescent detection systems for a Western blot has made it increasingly possible to identify proteins that were previously undetectable. Adaptation to a chemiluminescent detection system requires modifying and scaling down some of the factors involved in a Western blot. One common problem researchers encounter when improperly using this detection system is an occurrence commonly referred to as ghost bands. These bands appear as a halo with no signal in the middle of the band, or the entire band appears white in a dark background. To better guide researchers who use Pierce's highly sensitive SuperSignal<sup>®</sup> Chemiluminescent Substrates, we specifically addressed the above mentioned problems and determined some of their causes.

## Materials and Methods

### Cell Culture

A431 cultures (ATCC) were placed in 10 cm culture dishes in a humidified incubator at 37°C with 5% CO<sub>2</sub> and were grown to 80% confluency in DMEM medium (HyClone, Product No. SH30243-02) containing 10% FBS (HyClone, Product No. SH30070-03). Cells were treated with either 50 µM cisplatin for 1 hr and subsequently washed one time with phosphate-buffered saline (PBS) before supplementing with fresh DMEM medium. Cells were washed two times with cold PBS and lysed by incubating the plates for 15 min on ice with 250 µl of M-PER<sup>®</sup> Mammalian Protein Extraction Reagent (Product No. 78503) containing Halt<sup>™</sup> Protease Inhibitor Cocktail (Product No. 78410) and 1 mM EDTA. To obtain maximal yields of cellular proteins the cell lysate was also sonicated. Lysate proteins were then clarified by centrifugation at (7,500 × g) in a microcentrifuge for 10 minutes at 4°C and the supernatant stored as aliquots at -70°C. Protein concentration was estimated using the BCA Protein Assay (Product No. 23235).

### Western Blotting

Various amounts of purified NFκB protein (Upstate Biotechnology) or 30 µg of A431 lysates were separated on a 4-12% SDS-PAGE Bis-Tris gels (Invitrogen). Proteins were transferred to nitrocellulose membranes (Product No. 88025) in BupH<sup>™</sup> Tris-Glycine Buffer (Product No. 28380) containing 20% methanol for 1 hr at 100V. After transfer, membranes were blocked for 30 min at room temperature with either StartingBlock<sup>™</sup> Blocking Buffer (Product No. 37543) or non-fat dry milk (Nestlé Carnation) in Tris-buffered saline containing 0.05% Tween-20. The membranes were incubated overnight with either rabbit anti-NFκB antibody (1 mg/ml stock;

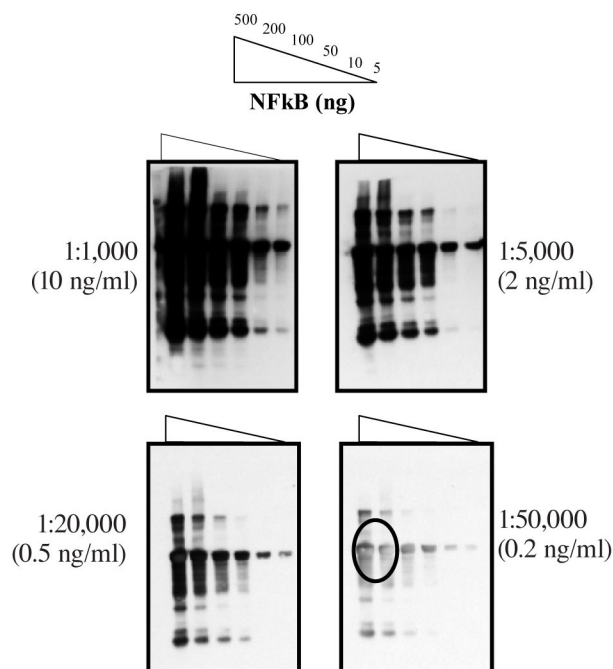
diluted 1:5,000; Upstate Biotechnology) or rabbit anti-p53 antibody (1 mg/ml stock diluted 1:1,000; GENEKA) and washed three times with phosphate-buffered saline (PBS). Goat anti-rabbit HRP conjugated secondary antibody (10 µg/ml stock diluted 1:1,000; component of Product No. 34076) was added to the blots and incubated for 1 hour at room temperature. Membranes were washed three times with PBS and 0.5 ml and 5.0 ml of either SuperSignal West Pico (SSWP, Product No. 34080) or SuperSignal West Dura (SSWD, Product No. 34076) Chemiluminescent Substrate was added and incubated for various periods. For detecting the chemiluminescent signal, membranes were exposed to CL-Xposure<sup>™</sup> Film (Product No. 34091) for various periods. To facilitate detection of two different proteins on the same membrane, the membranes were first probed with anti p53 antibody, then stripped using Restore<sup>™</sup> Western Blot Stripping Buffer (Product No. 21059) and re-probed with anti-NFκB antibody (Fig. 4, Panel A).

## Results and Discussion

In a Western blot, protein bands that have a halo appearance, regions that seem under-developed in the center of the bands, or white lanes in a dark background are commonly referred to as ghost bands. We determined that there are two main causes for such ghost bands: excess target protein (i.e., an overloaded gel) and cross-reaction of antibodies with components in the blocking solution.

Protein bands on membranes that were treated with 1:1000, 1:5000 or 1:20,000 dilutions (10 µg/ml stock) of secondary antibody were not significantly different from each other. However, 1:50,000 dilution (0.2 ng/ml) of secondary specifically enhanced the appearance of ghost bands. This effect was more evident in lanes that contained > 200 ng of NFκB (Fig. 1). Additionally, 64 hrs after completing the experiment, when intensity of the chemiluminescent bands was slightly reduced, ghost bands became more evident in all lanes having > 200 ng of NFκB, irrespective of secondary antibody dilution (data not shown).

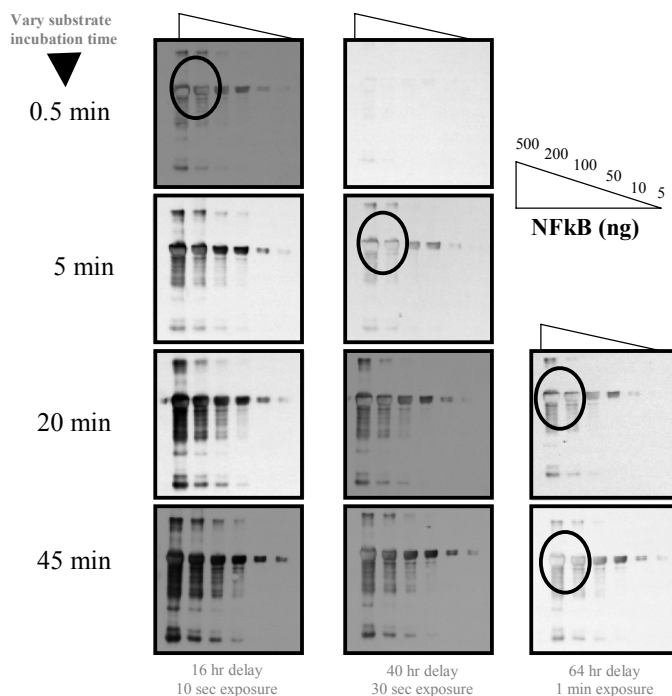
A substrate incubation time of 30 sec, which is considered sub-optimal, resulted in appearance of ghost bands in lanes containing > 200 ng of protein. Generally, longer incubation of the membrane in the substrate prolongs the duration of chemiluminescence signal. Using either SSWP or SSWD substrate enabled detection down to 10 ng of proteins even after 64 hours of completing the Western blot (Fig. 2).



**Figure 1.** Secondary antibody concentration affects the appearance of ghost bands. Western blots were performed on serially diluted NFKB proteins using various dilutions of secondary antibody. Ghost bands (oval) are present in lanes containing > 200 ng of NFKB. Membranes were incubated in SSWP for 5 min and then exposed to X-ray film for 30 sec.

SSWD is a more sensitive and longer lasting substrate than SSWP and other chemiluminescent detection system currently available. Although the SSWD produced significantly stronger signal than SSWP when detecting 5 ng of proteins, lanes with greater than 100 ng resulted in rapid signal loss. This experiment emphasizes the importance of using an appropriate amount of target protein when using highly sensitive detection systems. Rapid signal loss may result from enhanced catalysis of substrate by a high density of enzyme-conjugated secondary antibody that becomes bound to an excessive load of target protein, causing rapid depletion of the substrate in these regions of the blot. Note that membranes treated with SSWD were exposed to X-ray film 3 hr after substrate incubation because signal intensity was initially extremely high (Fig. 3).

A common method of blocking areas on a blot that do not contain transferred protein is by incubation with a non-fat milk solution. For the Western blotting conditions we tested, varying the concentration of non-fat milk in the blocking solution did not result in the appearance of ghost bands. Surprisingly, using as low as 0.5% non-fat milk in the blocking solution enhanced detection of low abundance proteins. We obtained similar results using either anti-NFKB crude serum or purified p53 antibodies (Fig. 4 panel A). However, cross-reaction of antibody with the blocking solution did result in the appearance of ghost bands. If the primary or secondary antibody binds to the milk proteins, these blocked sites chemiluminesce to produce a dark background with white lanes where sample proteins populate the membrane surface (Fig. 4 panel B).



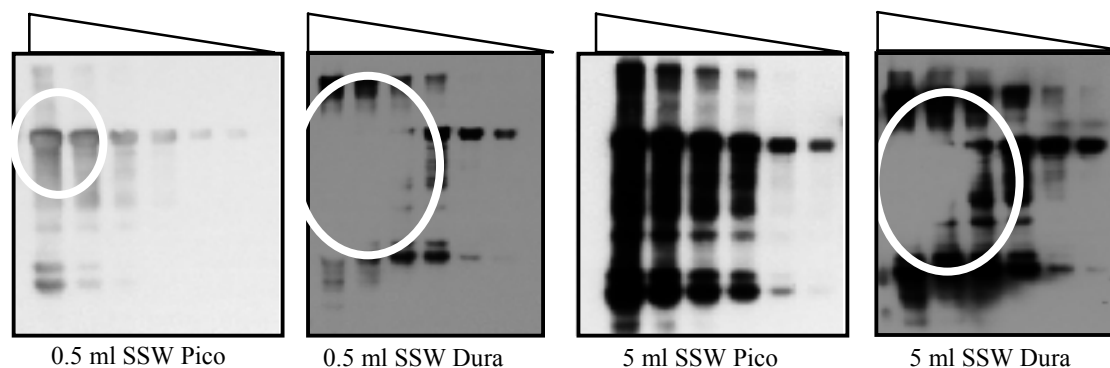
**Figure 2.** Protein amount and substrate incubation period affect signal duration. Western blots were performed on serially diluted NFKB using various incubation times with the chemiluminescent substrate. Note that a 45 min exposure of membrane to the substrate containing developing reagent could result in detection of as little as 10 ng even after 64 hours of the experiment. Because the exposure time to X-ray film was for one minute only the user may choose to perform a longer exposure to film to significantly enhance the detection of low abundance proteins. All membranes with > 200 ng of NFKB had a significant increase in the appearance of ghost bands (ovals) with decrease in signal intensity over a prolonged period.

## Conclusion

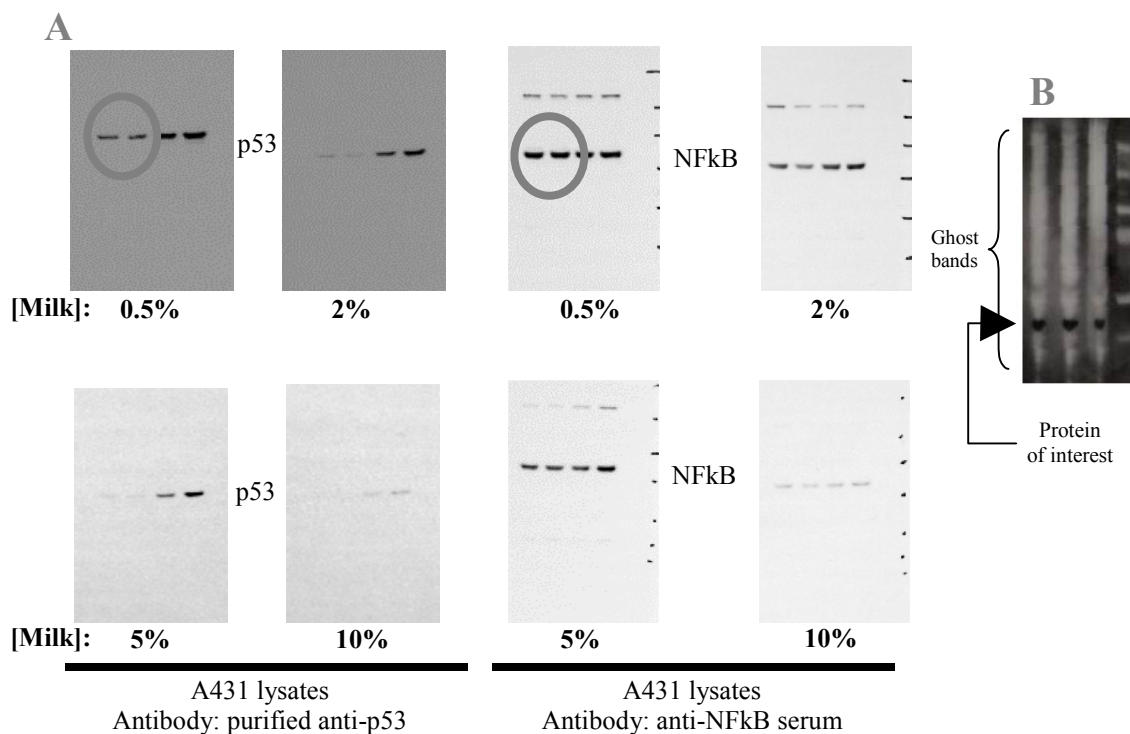
The chemistry that causes the appearance of ghost bands is unknown; however, it appears that high amounts of protein loaded on a gel also binds high amounts of HRP-conjugated secondary antibody to these regions. Enhanced catalysis and turnover of the substrate in these concentrated areas of immune complexes can result in a rapid depletion of the chemiluminescent substrate resulting in the appearance of ghost bands. In conclusion, excess loading of gels, high concentrations of secondary antibody, suboptimal incubation times with chemiluminescent substrate, and cross-reactivity of antibodies with blocked areas can cause the appearance of ghost bands. Optimization of Western blot parameters is especially critical to prevent ghost bands when using highly sensitive detection systems, such as SSWD and SuperSignal® West Femto Substrate.

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**Figure 3.** Excess loading of gels results in rapid loss of signal when using sensitive chemiluminescent detection systems. SSWD is a more sensitive and longer lasting chemiluminescent detection system compared to SSWP. The former reagent has significantly stronger signal when detecting 5 ng of proteins, however counter intuitively using high amounts of the proteins resulted in a rapid loss of signal. Also note that the membranes treated with SSWD were exposed to membrane 3 hours after the incubation with substrate since the signals were very high in the initial periods of time. The ovals on the left hand panels indicate the position of the ghost bands. The oval shapes on the right hand panels indicate the regions where there is a loss of signal.



**Figure 4.** Low concentration of non-fat milk (blocker) enhances the detection of low abundance proteins. Panel A: The indicated concentrations of non-fat milk were used for blocking and antibody incubation steps. Membranes were first probed with p53 antibody and subsequently stripped using Restore Western Blot Stripping Buffer to facilitate probing with NFkB antibody. The four lanes in each blot represent different cell harvest times following 1 hr treatment with 50  $\mu$ M cisplatin (0, 8, 20, 30 hr). Panel B: Membrane was incubated with 5% non-fat milk blocking buffer for 48 hr. The primary antibody used bound to the non-fat milk blocked regions on the blot, producing the appearance of ghost bands.